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FEBS Letters 337 (1994) 56–59

FEBS 13486

FEBS LETTERS

Mitochondrial ATP hydrolysis and ATP depletion in thymocytes and Ehrlich ascites carcinoma cells

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Received 1 November 1993

Abstract

When Ehrlich ascites carcinoma (EAC) cells and thymocytes were treated with uncoupler or rotenone in glucose-free medium, rapid ATP depletion was observed in both types of the cells. Oligomycin slowed down ATP loss in thymocytes, but not in EAC cells. Thus, mitochondrial ATP hydrolysis appears to be significant in deenergized thymocytes in contrast to EAC cells, in which other ATP consuming reactions were prevailing. Complete deenergization of mitochondria by uncoupler or rotenone in these cells resulted in inactivation of mitochondrial ATPase by 65–75%. The effect was observed after complete and rapid (20–30 s) disruption of the cells with detergent, Lubrol WX. ATPase was blocked by the specific inhibitor protein (IF_1) as it was shown by the studies on reactivation of this enzyme. When respiration is blocked but ATP content is supported by glycolysis, mitochondrial ATPase is not suppressed by IF_1, and maintains the energization of mitochondria. It is concluded that under complete de-energization of mitochondria IF_1 significantly inhibits mitochondrial ATP hydrolysis and may slow down ATP loss in thymocytes and EAC cells.

Key words: ATP depletion; Glycolysis, Mitochondrial ATPase; ATPase inhibitor protein (IF_1); EAC cell; Thymocyte

1. Introduction

Mitochondrial damage under various toxic and stress conditions induces ATP depletion, disturbance of Ca^{2+} homeostasis and cell death [1]. The depletion of ATP arises not only from cessation of oxidative phosphorylation but also from induction of ATP hydrolysis in uncoupled mitochondria. ATP hydrolysis in mitochondria can be prevented by the specific protein inhibitor (IF_1) [2]. This protein (MW ~ 10,000) does not affect the stationary rate of oxidative phosphorylation, but completely blocks ATP hydrolysis when one molecule of IF_1 is bound to the molecule of ATPase [3]. This effect in intact mitochondria is induced by deenergization [4,5]. The efficiency of inhibitor's action is different in mitochondria from various types of cells. It is higher in slow beating hearts (bovine, pig, rabbit) than in fast beating (mice, rat) [6]. IF_1 is unable to block more than 30% of ATPase in mitochondria from rat liver, but inhibits ATPase completely in rapidly growing hepatoma [5,7]. The regulation of mitochondrial ATPase in intact cells was studied in experiments with rat cardiomyocytes [8]. It was shown that uncoupler or inhibitors of respiration induced partial inhibition of ATPase that persisted after disruption of the cells. It was proposed that IF_1 was involved in this effect, but no evidence was presented. On the other hand, it was shown that in rat heart mitochondria the molar content of IF_1 did not exceed 30% of ATPase content [6].

In the present work we have studied the depletion of ATP and mitochondrial ATPase regulation in rat thymocytes and Ehrlich ascites carcinoma (EAC) cells which are extremely different in their energetics. In thymocytes the significant contribution of mitochondrial ATP hydrolysis in the process of ATP depletion induced by uncoupler was demonstrated. In EAC cells the hydrolysis is slow in comparison with the other ATP- consuming reactions. IF_1-dependent inhibition of ATPase was demonstrated in the both cell types. It reached 65–75% when mitochondria in the cells were completely de-energized with uncoupler.

2. Materials and methods

Thymocytes were obtained from white rats as described [9] in the following medium: 142 mM NaCl, 5.6 mM KCl, 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$, 8 mM MOPS, 10 mM glucose, pH 7.4. Ehrlich ascites carcinoma cells (EAC) were cultivated in Swiss albino mice and were obtained on 6–8 day after inoculation using the same medium. The cells were washed thrice in above-mentioned medium and stored at 10^9 and 0.2×10^9 cells per ml for thymocytes and EAC cells respectively on ice.

The cells were incubated at 7×10^7 (thymocytes) and 0.7×10^7 (EAC

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Abbreviations: EAC, Ehrlich ascites carcinoma; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

cells) per ml in the medium contained 142 mM NaCl, 5.6 mM KCl, 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$, 50 mM MOPS, pH 7.4, with or without 10 mM glucose at 37°C. Cell respiration was measured by Clark oxygen electrode; lactate production with lactate dehydrogenase and ATP content with luciferin-luciferase as described [10]. For the measurement of ATPase activity cells were disrupted with 0.2% Lubrol-WX in the incubation medium contained additionally 3 mM EDTA for 30 s. The process of disruption was controlled by light microscope. ATPase reaction was initiated by addition of cell extract (20 μl) to 0.5 ml of the medium contained 20 mM KCl, 20 mM Tris, 1 mM EDTA, 10 mM Na_2SO_3, 2 mM ATP, 4 mM $MgSO_4$, 1 μM CCCP, pH 8.2. After 10 min incubation at 37°C reaction was terminated with 5% SDS and 10 mM EDTA and P_i was measured by [11]. Na_2SO_3 in the medium improved the linearity of P_i accumulation and increased oligomycin-sensitive ATPase activity by approximately 30%. Oligomycin-insensitive ATPase activity was determined in every sample in the presence of 2 μg/ml oligomycin and varied from 54% to 61% of total activity in thymocytes and from 37% to 42% in EAC cells.

Reactivation of ATPase was studied after preincubation and disruption of the cells as described above. The reactivation medium contained 20 mM KCl, 20 mM Tris, 1 mM EDTA, 1 μM CCCP pH 8.2 or 7.4 at 37°C.

3. Results and discussion

3.1. ATP depletion and mitochondrial ATP hydrolysis in the cells

In thymocytes inhibition of oxidative phosphorylation by rotenone or uncoupler (CCCP) induced rapid ATP depletion. This process was slightly slowed down in the presence of glucose. Oligomycin partially protected cellular ATP both in the presence and in the absence of glucose (Table 1). These data indicate that mitochondrial ATP hydrolysis plays an important role in ATP depletion induced by uncoupling of oxidative phosphorylation in this type of cells.

Table 1

ATP depletion under de-energization of mitochondria in EAC cells and thymocytes

Conditions of incubation	ATP content (% of initial)			
	15 min incubation		30 min incubation	
	EAC cells	Thymocytes	EAC cells	Thymocytes
(1) No addition	62 ± 9	94 ± 6	71 ± 7	75 ± 2
(2) Rotenone (2 μM)	5.1 ± 0.6	8.5 ± 0.5	3.3 ± 0.4	9.0 ± 2.0
(3) CCP (2 μM)	6.3 ± 0.7	8.7 ± 0.7	4.9 ± 0.1	8.2 ± 1.2
(4) CCCP (2 μM) + Oligomycin (2 μg/ml)	6.8 ± 1.2	17.5 ± 2.6	4.6 ± 1.0	13.8 ± 1.3
(5) Glucose (10 mM)	106 ± 7	94 ± 6	118 ± 24	93 ± 7
(6) Glucose (10 mM) + Rotenone (2 μM)	84 ± 6	19.6 ± 3	90 ± 20	12.0 ± 3.8
(7) Glucose (10 mM) + CCCP (2 μM)	96 ± 15	20.4 ± 4.6	70 ± 5	12.8 ± 2.7
(8) Glucose (10 mM) + CCCP (2 μM) + Oligomycin (2 μg/ml)	n.d.	42.5 ± 2.5	n.d.	29.1 ± 0.5

Data are mean values ± S.E.M. of 3–5 experiments. Initial ATP content (before incubation) was 25.5 ± 1.5 nmol/10^6 cells in EAC cells and 1.6 ± 0.2 nmol/10^6 cells in thymocytes. n.d. = not determined.

Table 2

Stimulation of glycolysis by rotenone and CCCP in EAC cells and thymocytes

Conditions of incubation	Lactate production (nmol/min × 10^6 cells)			
	EAC cells		Thymocytes	
(1) No additions	4.5 ± 0.18	(100%)	0.077 ± 0.005	(100%)
(2) Rotenone (2 μM)	8.9 ± 0.6	(198%)	0.180 ± 0.02	(233%)
(3) CCCP (2 μM)	11.3 ± 1.0	(251%)	0.178 ± 0.014	(231%)
(4) Rotenone (2 μM) + Oligomycin (2 μg/ml)	7.7 ± 0.7	(171%)	0.178 ± 0.004	(231%)
(5) CCCP (2 μM) + Oligomycin (2 mg/ml)	6.9 ± 0.4	(153%)	0.190 ± 0.023	(246%)

Data are mean values ± S.E.M. of 3–5 experiments. Lactate production was determined in the presence of 10 mM glucose during first 15 min (EAC cells) or 30 min (thymocytes) of incubation at 37°C.

In EAC cells uncoupler and rotenone only partially (20–30%) decreased the ATP content in the presence of glucose (Table 1) obviously due to the high rate of aerobic glycolysis in these cells in contrast to thymocytes (Table 2). After initial dropping ATP in EAC cells was stabilized and remained constant during one hour at least (not shown). The consumption of ATP in this case was equilibrated with the glycolytic ATP synthesis. Actually, rotenone (2-fold) and CCCP (2.5-fold) stimulated lactate production in EAC cells (Table 2). Oligomycin inhibited glycolysis both in the presence of rotenone or uncoupler (Table 2). Since oligomycin did not change significantly the stationary ATP concentration in the presence of CCCP (not shown), the rate of oligomycin-sensitive glycolysis coincides with the rate of mitochondrial ATP hydrolysis. This rate was equal to 4.4 nmol/min 10^6 cells and corresponded to approximately 40% of the total rate of ATP consumption in EAC cells.

In the absence of glucose rotenone and CCCP induced ATP depletion in EAC cells to the same extent as in thymocytes. Oligomycin practically did not protect cellular ATP (Table 1), so ATP depletion under these conditions was determined by the nonmitochondrial ATP-consuming processes. It can be proposed that glucose deprivation inhibits mitochondrial ATP hydrolysis and/or stimulates the other ATP-consuming reactions in EAC cells.

3.2. Inactivation of mitochondrial ATPase in the cells

Incubation of thymocytes and EAC cells in the presence of rotenone or CCCP resulted in inactivation of mitochondrial ATPase (Table 3). The effect was observed after complete and rapid (20–30 s) disruption of the cells with detergent – Lubrol WX. We have shown

earlier [5] that Lubrol did not disturb either the structure of F_0F_1-ATPase or its interaction with the inhibitor protein in mitochondria from liver and hepatoma. This treatment, however, completely abolished the transport limitations and allosteric (reversible) regulation. The disruption of the cells did not induce any additional IF_1-dependent inhibition. This was confirmed in experiments on ATPase reactivation that did not reveal inactive IF_1·ATPase complexes in untreated cells (see next section). The reactivation of ATPase during disruption also was not significant. Actually, when MgATP (that completely prevented any reactivation, see below) was added to the disruption medium, no changes in ATPase activity of the cells preincubated with CCCP were observed (not shown).

Inactivation of ATPase induced by uncoupler was more pronounced in EAC cells (74%) than in thymocytes (65%). This difference may be partially caused by lower pH in cytoplasm of ascite tumor cells, that promoted inhibitor's action [2]. In the absence of glucose, when pH in EAC cells was higher, the effect of uncoupler was slightly suppressed (Table 3).

Rotenone, that completely blocked respiration in the both types of cells, induced significantly less inhibition of ATPase than uncoupler (Table 3). This difference was more pronounced in EAC cells in the presence of glucose, when ATP content in the cell was high (Table 1). In thymocytes incubated in the absence of glucose, rotenone induced the decrease of ATP content and ATPase inhibition practically to the same extent as CCCP. These data indicate that the maximal inhibition was reached at complete deenergization of mitochondria. When respiration was blocked, but ATP content was supported by glycolysis, mitochondrial ATPase ensured partial energization that prevented IF_1-dependent inhibition of the enzyme.

Both in thymocytes and in EAC cells inactivation of

Table 3

Effect of CCCP and rotenone on oligomycin-sensitive ATPase activity in EAC cells and thymocytes

Conditions of incubation	ATPase activity (nmol ATP/min × 10^6 cells)			
	EAC cells		Thymocytes	
(1) No additions	77.1 ± 1.1	(100%)	5.03 ± 0.11	(100%)
(2) CCCP (2 μM)	23.9 ± 1.1	(31.0%)	1.76 ± 0.07	(35.0%)
(3) Rotenone (5 μM)	49.8 ± 0.8	(64.6%)	2.09 ± 0.10	(41.6%)
(4) Glucose	82.0 ± 1.3	(100%)	5.30 ± 0.11	(100%)
(5) Glucose + CCCP (2 μM)	21.3 ± 1.0	(26.0%)	1.88 ± 0.11	(35.5%)
(6) Glucose + Rotenone (5 μM)	61.8 ± 1.7	(75.4%)	3.40 ± 0.16	(64.2%)

Data are mean values ± S.E.M. of 6 experiments. EAC cells and thymocytes were incubated (see section 2) for 15 min in the presence or in the absence of glucose (10 mM) and for 15 min with CCCP or rotenone.

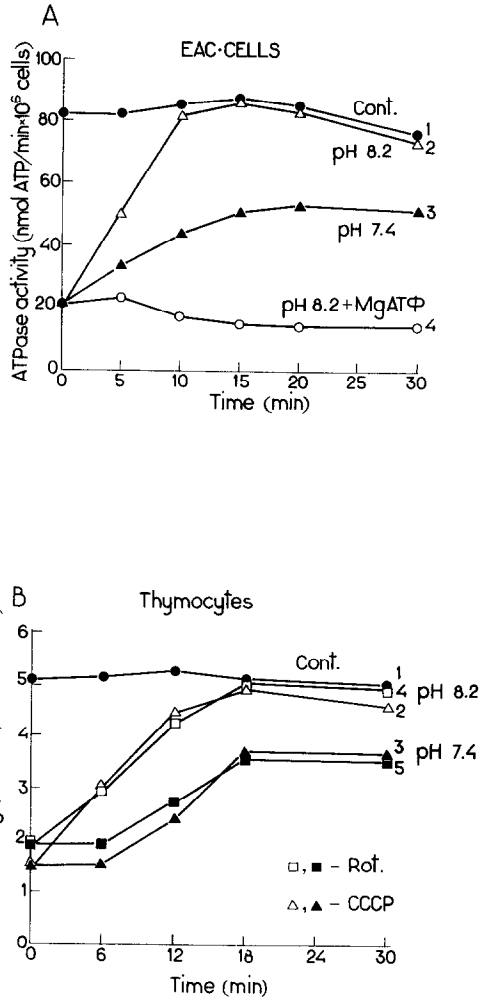

Fig. 1. Reactivation of mitochondrial ATPase in EAC cells (A) and thymocytes (B). Cells were preincubated in the presence of glucose as described in Table III without additions (curves 1A, 1B); with 2 μM CCCP (curves 2A, 3A, 4A, 2B, 3B) or with 5 μM rotenone (curves 4B, 5B). After disruption with 0.2% Lubrol (30 s), cell extracts were transferred to the reactivation medium (see section 2) at pH 8.2 (curves 1A, 2A, 4A, 1B, 2B, 4B), or at pH 7.4 (curves 3A, 3B, 5B). At the indicated times pH was adjusted to 8.2 when necessary (curves 3A, 3B, 5B) and ATPase reaction was initiated with 4 mM $MgSO_4$, 2 mM ATP and 10 mM Na_2SO_3. Curve 4A: 4 mM $MgSO_4$, 2 mM ATP, 10 mM K_2SO_3, 5 U/ml pyruvate kinase and 2 mM phosphoenolpyruvate were added to the reactivation medium and P_i liberation at indicated times was measured.

ATPase was not complete even in the presence of uncoupler. The residual ATPase activity was not an artefact of disruption and measurement procedures. In intact EAC cells the rate of mitochondrial ATP hydrolysis in the presence of uncoupler and glucose was determined (see above). This value – 4.4 nmol ATP/min 10^6 cells – was lower than ATPase activity measured after disruption (21.3 nmol ATP/min 10^6 cells). The difference was probably the result of ADP-dependent inhibition of ATPase in intact cell. It was shown earlier [12] that nearly 90% of ATPase formed an inactive complex with

MgADP in intact liver mitochondria during uncoupled ATP hydrolysis.

3.3. Reactivation of mitochondrial ATPase

The inactivation of ATPase by CCCP and rotenone was slowly reversible (Fig. 1). The complete reactivation was observed if the cells were disrupted and incubated at high pH (8.2 or higher) in the presence of EDTA (curves 2A, 2B and 4B). The reactivation was slower at lower pH (curves 3A, 3B and 5B) and was completely prevented by MgATP (curve 4A). The reactivation of IF_1-ATPase complex in submitochondrial particles from beef heart possesses the same features [13]. These data indicate that the inactivation of ATPase which is induced by uncoupler or rotenone and persisted after disruption of the cells is completely the result of inhibitor protein's action.

In untreated cells incubated both in the presence or in the absence (not shown) of glucose no reactivation of ATPase after disruption was observed (Fig. 1, curves 1A, 1B). These data demonstrate that IF_1 blocks the enzyme only after deenergization of mitochondria. The fraction of de-energized mitochondria in thymocytes and in EAC cells is very small both at high and low rate of glycolysis. It is interesting to compare these results with the data on mitochondrial ATPase regulation in cardiomyocytes [8]. In the resting cardiomyocytes approximately 50% of ATPase is blocked and can be activated upon electrical stimulation of the cells. The mechanism of the inhibition was not determined in these experiments but the participation of the inhibitor protein was proposed.

4. Conclusions

The data presented here demonstrate that ATP is hydrolysed by mitochondrial ATPase in living cells in the presence of uncoupler or respiratory inhibitor. In thymocytes this futile hydrolysis accelerates ATP depletion. In EAC cells it is slow in comparison with the other ATP-consuming processes.

It can be proposed that the low rate of hydrolysis is a result of inhibition of mitochondrial ATPase by the specific inhibitor protein (IF_1). IF_1-dependent inhibition is observed in the both cell types and reaches maximum (65–75%) when mitochondria are completely de-energized with uncoupler. When respiration is blocked but ATP content is supported by glycolysis (in EAC cells), mitochondrial ATPase is not blocked by IF_1, and maintains the energized state that may be of physiological significance. At high level of energization (in untreated thymocytes and EAC cells) inactive complex of ATPase with IF_1 is not formed.

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